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CONTRACT NO: DAMD17-94-C-4037

TITLE: PARASITE LACTATE DEHYDROGENASE FOR THE DIAGNOSIS OF PLASMODIUM FALCIPARIUM

PRINCIPAL INVESTIGATOR: Robert C. Piper, Ph.D.

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REPORT DATE: August 8, 1994

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13. ABSTRACT (Maximum 200 words)

We have developed a rapid and quantitative method for the detection of all species of malarial parasties that infect humans. The assay is based on a patented process that can specificallly measure *Plasmodium sp.* Lactate dehydrogenase (pLDH). The assay is reproducible and has been standardized with the use of recombinantly expressed pLDH. The sensitivity of the original patented process has been greatly enhanced with the use of dye ligands and antibodies that specifically recognize pLDH. These reagents have been used to rapidly capture the pLDH enzyme and facilitate separation of the enzyme from factors that contribute to background signal. Our current pLDH technology can be formatted as either a "wet" assay or a "dry" dip-stick test that can measure pLDH activity form either whole blood, Red Blood Cells, or serum/plasma. This technology may also be applied to measure the drug-sensitivity of parasites isolated from individual patients. Our current technology, the pLDH test is now ready for verification in the field.

14. SUBJECT TERMS
MALARIA, *PLASMODIUM SP*, DIAGNOSIS, LACTATE DEHYDROGENASE, CULTURE AND SENSITIVITY, DRUG DEVELOPMENT, DRUG RESISTANCE, *FALCIPARUM*

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Final Report Phase I

Contract # DAMD 17-94-C-4037 <u>**Parasite Lactate Dehydrogenase for Diagnosis of *Plasmodium falciparum***</u>

I. Section 1- **Introduction**

The diagnosis of *Plasmodium sp.* has traditionally been performed by microscope. It is our intent to utilize the SBIR to develop a method to allow the quantification of the presence of the malaria parasite by the specific measurement of the parasite lactate dehydrogenase (pLDH). Flow inc. has patented a pLDH assay (US. #5124141 issued on June 23 1992).
The described work for our SBIR grant entitled "Parasite Lactate Dehydrogenase for Diagnosis of *Plasmodium falciparum.*" was to devise a method for the diagnosis of malaria in patient blood samples (see Gnatt Chart A). The test was to be based on the specific detection of a parasite enzyme, lactate dehydrogenase (pLDH), as a marker of active parasitic infections. Flow Inc. has patented a pLDH assay (US. #5124141 issued on June 23, 1992). Our goal was to format a "wet chemistry" test in a 96 well plate that could accurately and rapidly measure parasitemias of 0.005-0.01%. Our second goal was to formulate a "dip-stick" format for the pLDH assay using dry chemistry.

During the week of April 5th both Dr. Makler and Dr. Piper of Flow Inc. attended a scientific conference sponsored by the Rockefeller Foundation in Bellagio, Italy. During this time the utility of the pLDH assay was discussed among scientists from many different fields. During this meeting it was stressed that the utility of a simple "positive/negative" diagnostic test for malaria may not be useful in all situations. Furthermore, it was generally felt that it would be a of great clinical and epidemiological utility to develop a way of obtaining a drug-sensitivity profile for individual malarial infections in a simple and timely manner. We believe that the pLDH assay coupled with a consistent short-term culture technique of parasites isolated from patient's blood could be developed to meet this need. Such a test would follow the development of the early ring-stage malarial parasite to the trophozoite and schizont stage by measuring an increase in pLDH activity. Our preliminary data indicate that as the parasite progresses along this developmental pathway, more pLDH is produced per infected RBC. Thus, the development of the parasite and its sensitivity to fast acting drugs like chloroquine could be

measured in short-term cultures (24-36 hours) by a rapid and easy to perform enzyme assay. Such a drug-sensitivity test would be valuable to the clinician as a prognostic indicator of drug-resistant malaria infections in individual patients. This information, if timely, could drastically affect the therapeutic strategy. Furthermore, the drug sensitivity profiles of malarial infections could be standardized and aid in the overall surveillance of drug resistance wherever such tests were deployed.

We therefore request to change our original statement of work from the development of a simple positive/negative diagnostic assay to the initial development of a field-capable drug-sensitivity assay for the evaluation of individual infections of *Plasmodium falciparum*. The work proposed was to enhance the assay by implementation of a standard curve as well as produce an enzyme "capture" technique that could enhance specificity of the assay. We also evaluated the Malstat™ reagent with many clinical samples and took the initial steps to develop a "dip-stick" format for the pLDH assay.

II. Section 2. **Progress report**

Administration.
The staff at FLOW Inc. that have worked under the SBIR CONTRACT NO. DAMD 17-94-C-4037 "Parasite Lactate Dehydrogenase for Diagnosis of *Plasmodium falciparum"* are listed below:

Dr. Piper: principal ivestigator. Dr. Piper has been responsible for the cloning, expression and purification of pLDH and has raised mouse antibodies to the pLDH. During the work period Dr. Piper has spent 1 week in Bellagio, Italy for a conference entitled "The role of the glycolytic pathway in diagnosis and potential therapy for malaria " co-sponsered by FLOW Inc. and the Rockefeller Foundation. Dr. Piper was also invited to attend a Gordon Research conference, "Molecular Cell Biology" in Tilton, NH. the week of June 20.

Dr. Makler: President of FLOW Inc. Dr. Makler was retained as the priniciple consult on the project and has contributed to the evaluation of clinical samples and the formatting and quality control of the Malstat™ reagent. Dr. Makler also spent 10 days preparing for a business trip to ET to demonstrate the culture and sensitivity method developed under SBIR grant to Dr. Nuzum and Dr. Miller. The meeting went well as the both principle and practice of the c/s assay as prescribed by FLOW Inc. were validated. The purpose of meeting was to promote and facilitate the initial use of the c/s assay by ET personnel.

Ms. Wentworth: Laboratory technologist. Ms. Wentworth has been hired as a full time technologist and works directly with the diagnostic methods and

evaluation of clinical samples. Ms. Wentworth was away on vacation during the week of June 20.

Ms. Williams: is responsible for daily maintenance of malaria cultures.

Ms. Ries and Mr. Brown are involved in statistical evaluations, in performing culture techniques microsopic analysis and back up for *in vitro* culture techniques. Jean Williams is responsible for maintaining parasite cultures.

Dr. Oduola: Consult. Dr. Oduola from Nigeria is evaluating the prototype wet diagnostic assay on stored samples of red cells and plasma he has accumulated. He has also provided FLOW Inc. with many clinical samples to evaluate using the pLDH assay.

Research Activities

In accordance with both the old and the new Statement of Work submitted, the work at FLOW Inc. has been focused on the followig areas:
1) cloning and expression of pLDH in heterologous systems
2) Establishing a standard curve for pLDH activity using the recombinant pLDH
3) evaluation of clinical samples with the Malstat™ reagent
4) Preliminary formatting of a "dry" dip-stick format for the pLDH diagnostic assay
5) Production of anti-pLDH antibodies for use in a more sensitive capture assay.

Project 1: Expression of pLDH
A PCR fragment encompassing the entire open reading frame was amplified from *Plasmodium* DNA and subcloned into the yeast expression vector pUT-102 which expresses pLDH via the Alchohol Dehydrogenase promoter. The resulting plasmid was transformed into the yeast *Saccharomyces cerevisiae*. Yeast spheroplasts were then lysed and the soluble pLDH activity purified over Cibacron Blue sepharose. The yeast *Saccharomyces cerevisiae* was chosen since no endogenous yeast LDH is produced. Therefore, the only LDH activity in the yeast lysate is that of recombinant pLDH. This allowed us to work with crude lysates if necesssary. A large amount of the yeast recombinant pLDH (yrpLDH) was purified and biochemically analyzed both at FLOW Inc. and by Dr. Vander Jagt at the University of New Mexico under the terms of a collaborative investigation. We find that yrpLDH has **identical** biochemical properties to authentic pLDH isolated from infected red blood cells.
We have also produced recombinant pLDH in the bacteria *E. coli* (brpLDH) using a bacterial expression system. BrpLDH can be produced much more

cheaply and much more easily than the yrpLDH. Our purification procedure is very effective for purifying pLDH from other endogenous bacterial dehydrogenases. The specific activity of recombinant pLDH per mg protein is approxiamately 20 fold higher in the bacterial system vs. the yeast system. The Km and Vmax for the brpLDH is identical to that of authentic pLDH. There is a very small difference in the electrophoetic mobility of brpLDH on non-denaturing agarose electrophoresis gels and we are currently investigating the reason for this slight difference. Currently, we believe this to be caused by a difference in N-terminal acetylation which has no effect on enzyme activity. However, the brpLDH is stable and appropriate for incorporation in standard curves as well as future structure/function studies. An example of the two recombinant pLDH preparations is shown in Figure 1. (The bacteria system overproduces pLDH to such an extent that some of the enzyme is left at the application origin)

Project 2: Establishing a Standard Curve.
Using the yrpLDH produced at FLOW Inc. we have established a standard curve for calibrating the measurement of pLDH in clinical samples. Typically we have a set of enzyme dilutions that is run in parallel with samples of parasitized blood. The pLDH activity in the clinical sample is then matched with that generated by the yrpLDH dilutions. An example of such a standard curve is given in Figure 2. We have reproduced an earlier curve and find that a **1% parasitemia has ~110 Units pLDH/liter; or ~2nU pLDH/infected RBC.** The range of specific pLDH acivity in clinical samples of *P. falciparum, P. malariae, P. vivax,* and *P. ovalae* obtained is being evaluated in clinical samples. We find these levels to be comparable, however, we have not had access to many of these types of samples to have a rigorous answer. It is important to stress that the standard curve has been made with pLDH rather than an LDH isoform from some other organism which has some reactivity with Malstat™ reagent. This not only allows a more accurate calibration of pLDH activity but more importantly, it allows the standard curve to work in a "capture" assay format that utilizes antibodies specific for pLDH.

Project 3: Evaluation of Clinical Samples
Ms. Wentworth, utilizing the wet assay, has analyzed over 300 stored samples of plasma and red cell from Africa and Central America in collaboration with Dr. Oduola. The analysis has pointed out some of the limitations of sample storage, the need for more precise timing for color development in the wet assay when utilizing plasma. In addition, these preliminary studies have provided some indication of the intra and inter-assay variations of the pLDH diagnostic method; and enabled a comparison of the equivalency of plasma and red cell hemolysates for pLDH activity and also reemphasized the need to improve the sensitivity of the assay by incorporating a capture step for the pLDH.

In spite of these limitations, Ms. Wentworth has completed a study of over 30 clinical samples contributed from primates with malaria from the CDC. These results have been compared and tabulated. An abstract is in preparation. Ms Wentworth has also performed numerous quality control experiments for the co-enzyme analog APAD from various commercial sources of the analog and different sources of diaphorase. In addition she performed electrophograms on many of the clinical samples from Colombia and from Kenya as well as from the CDC. The pLDH assay was evalauted with blood samples containing primate malaria . Ms. Wentworth has also continued her studies to move the chemistry of the pLDH assay to a dry format. These studies have focused mainly on the elimination of hemoglobin from the site of pLDH-dependent blue color formation(see figure). A large number of samples from Panama were analyzed during May by the pLDH assay as well as electrophoresis.

Project 4: Preliminary Formatting of a "dry" dip-stick format for the pLDH diagnostic assay.

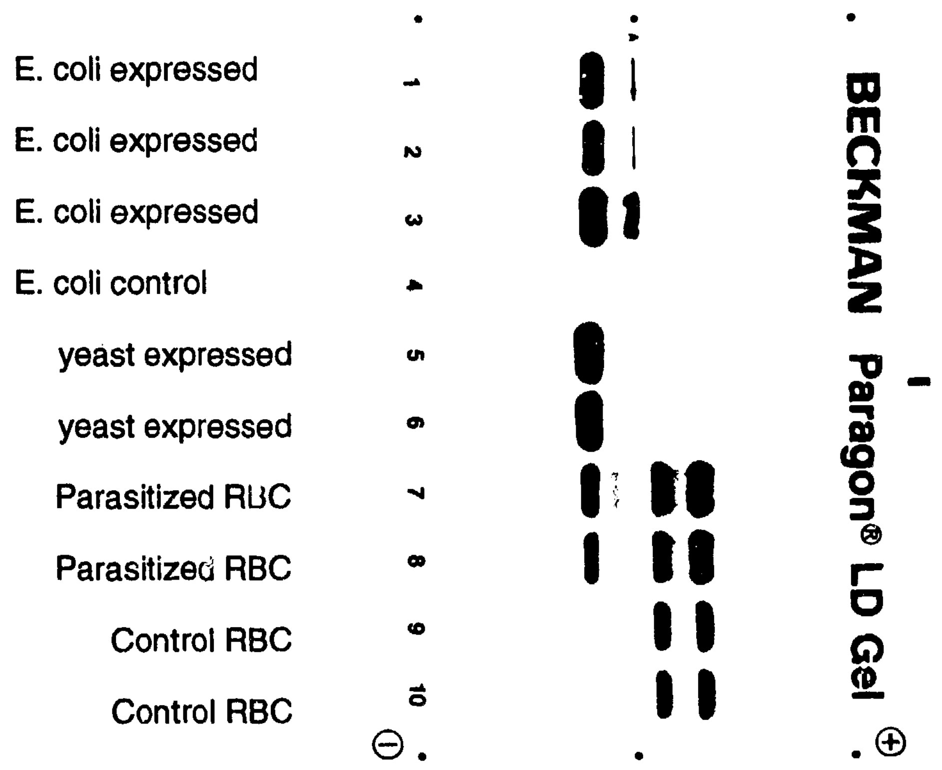
Development of the "dry" format was only possible by first eliminating the background produced by the PES/NBT reaction that converts APADH into a blue formazan salt. Studies with the use of the enzyme diaphorase as a replacement for PES have shown that diaphorase is a preferred catalyst. Several diaphorases have been found that can achieve this goal; however, one in particular has a preference. Arrangements are now being made to acquire significant amounts of these enzymes to evaluate the use of diaphorase in a standardized format.

Studies were also done to simulate a future pLDH capture assay. Instead of using anti-pLDH antibodies we used Cibacron Blue sepharose. This matrix can reversibly bind LDH enzymes at the active site and was used to separate LDH from hemolysates from a variety of parasite-infected samples to simulate the procedures to be employed with the eventual antibody capture methods. As a test of principle Ms. Wentworth constructed a "dip" stick in which the Cibacron Blue was attached at one end of a strip of filter paper. Infected blood was then apllied and left on for 2 minutes. Water or 20 mM Tris was then added which caused the red hemoglobin to migrate away from the sample aplication site. A mixture of Malstat™, diaphorase, and NBT was added and the captured pLDH activity converted the NBT to a dark blue color. This separation procedure increased the signal to noise ratio primarily by reducing the background reaction caused by hemaglobin. This

separation technique represents one way to apply the monoclonal anti-pLDH antibodies we are currently developing.

Project 5: Production of anti-pLDH antibodies for use in a more sensitive capture assay.
To increase the sensitivity and versatility of the pLDH assay we have chosen to develop a panel of monoclonal antibodies specific for pLDH. These antibodies can be incorporated into an enzyme capture assay that would enrich pLDH in either a wet or dry assay format. We also hope that we may find antibodies that can distinguish pLDH from different species of human trophic *Plasmodium*. We have now successfully immunized 8 Balb-C mice against pLDH and obtained polyclonal antibodies that can capture pLDH activity. Originally, we produced a fusion protein for the immunogen composed of the Glutathione-S-transferase protein fused to the entire open reading frame of pLDH. This fusion protein was purified but is not used as antigen because it did not represent an active protein. To help ensure we could obtain antibodies that would recognize active pLDH we instead decided to immunize with highly purified yrpLDH. The mice were immunized and boosted 2 times. Polyclonal antiserum was collected and attached to Protein G sepharose which was then used to capture pLDH. All 8 serums contained antibodies that could capture both yrpLDH and brpLDH (Figure 4). Preimmune serum captured **no activity** demonstrating the specificity of this technique. We are now in the process of producing monoclonal antibodies from these immunized mice.

FIGURE 1) Analysis of brpLDH and yrpLDH



Recombinant pLDH produced in bacteria or yeast was subjected to agarose electrophoresis and stained with Lactate, NAD, NBT and PES to show all LDH isoforms. Bacteria with vector alone is shown as bacteria control. The specificity of the yeast recombinant system has been given in a previous report.

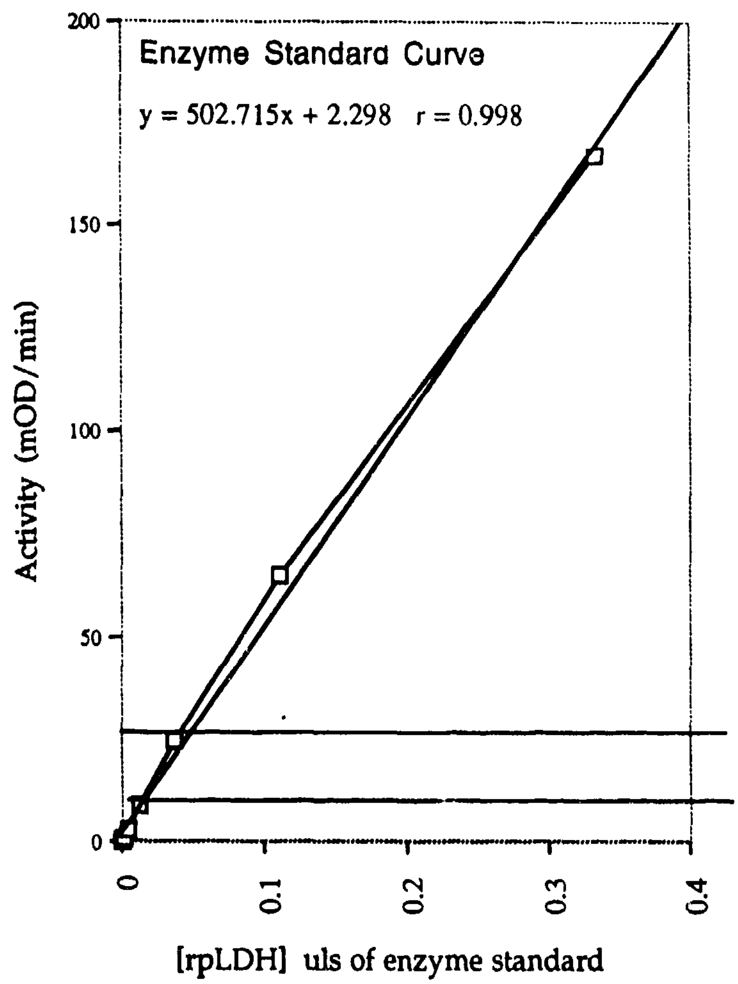
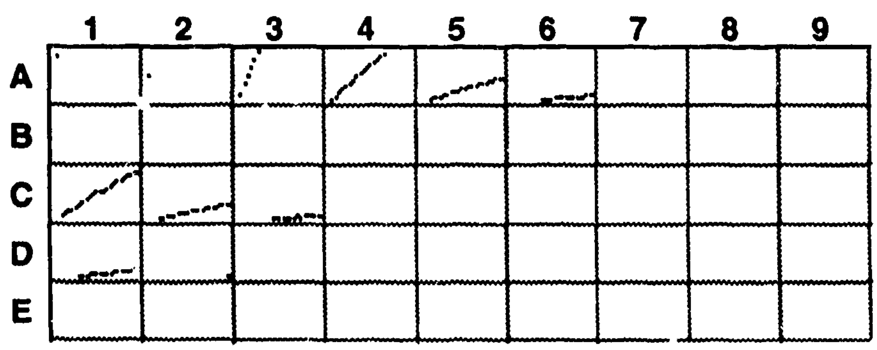
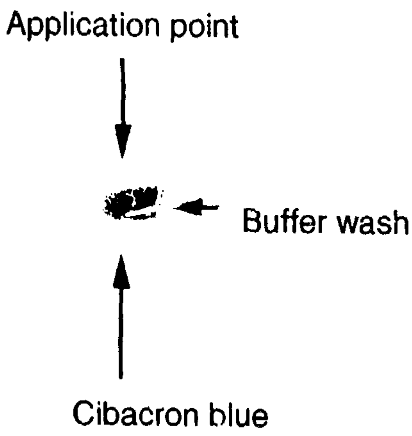


Figure 2) Standard Curve of pLDH Purified rpLDH was serially diluted 1:2 and run with 200 uls of Maistat™ in the "A" row shown below. The mOD/min are plotted as a function of enzyme concentration. The correlation coefficient was 0.998. Samples of parasitized RBC were subjected to four serial dilutions (1:2) and run in row "C" (0.5% parasitemia) row "D" (0.1% parasitemia) and row "E" (control RBC). The activity measured in the parasitized cells is proportional to the starting parasitemia. Enzyme activity was measured with a Molecular Devices microtitre plate reader. Measurements are given below.



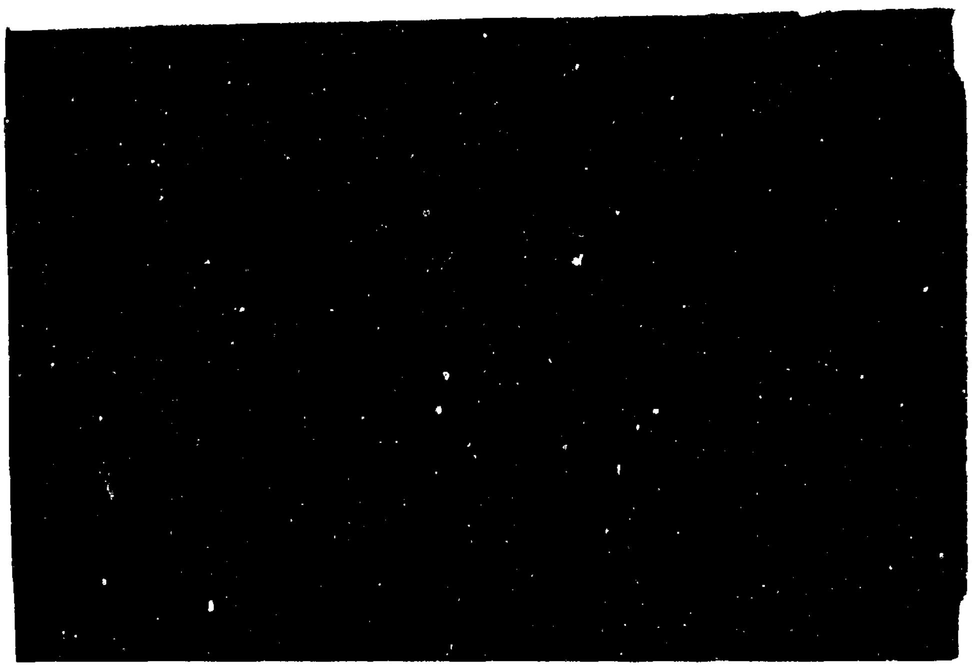
	1	2	3	4	5	6	7	8	9
A	232.0	167.6	65.50	24.75	8.800	2.812	0.727	0.388	-0.024
B									
C	18.50	6.545	2.036	0.800					
D	3.733	1.188	0.339	0.073					

Figure 3) Capture of pLDH on a "dip-stick"



Cibacron blue was applied to Etcher 31 chromatography paper and soaked in Briggs detergent. Parasitized RBC were then applied at the origin and cell lysing and binding of pLDH was allowed to proceed for 3 minutes. A buffer wash was then applied that caused the hemoglobin to migrate away from the origin. Color was developed with Malstat™, NBT and Diaphorase applied to the origin

Figure 4) Capture of pLDH by mouse polyclonal anti-pLDH antibodies



8 immunized balb-c mice were bled and the resulting sera were attached to protein-G couple sepharose. Sepharose was washed 3 times in PBS and either yrpLDH or brpLDH from crude extracts was added to the beads for 5 minutes. Beads were then washed 2 times in PBS and 10 uls of beads were placed in a microtitre plate containing 200 uls of Malstat™ with NBT/PES. The reaction was arrested after 4 minutes with the additio of acetic acid.

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